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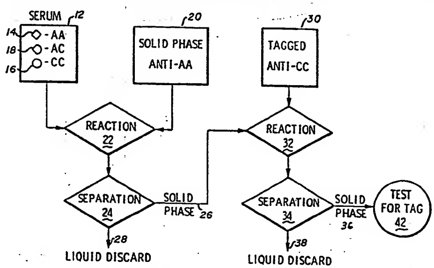
(54) Title: TWO SITE ENZYME LABELED CROSS REACTION IMMUNOMETRIC SANDWICH ASSAY METHOD



(57) Abstract

A two site cross-reaction immunometric sandwich assay method for the detection and measurement of an analyte, such as creatine phospho-kinase-MB, in serum comprising the selection of two different antibodies each of which is specific to a different analyte but each of which will cross-react with the analyte of interest. The first antibody is reacted with the unknown sample utilizing a solid-phase to bind the first antibody. Separation of the solid and liquid porti ns of the first reaction is accomplished and the solid portion thereof is reacted with the second antibody which is tagged. The solid portion and liquid portion of the second reaction are separated and the solid portion is tested for the tag as an indication of the presence of said analyte. With particular reference to testing for creatine phospho-kinase-MB in human serum, the cross-reacting antibodies utilized are antibody to creatine phospho-kinase-BB and creatine phospho-kinase-MM, and an enzymatic tag is utilized.

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Specification

Two Site Enzyme Labeled Cross Reaction Immunometric Sandwich Assay Method

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part to U.S. Patent Application Serial No. 06/165,001, filed 6/30/80.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to immunological assay techniques for determining the presence of an analyte in serum and more particularly to a two site cross-reaction immunoassay sandwich testing method which has particular application in the qualitative and quantitative determination of the level of the creatine phospho-kinase MB Isoenzyme (CK-MB) in human serum utilizing an enzyme label.

Description of the Prior Art

Conventional immunometric techniques depend upon the immunochemical reaction between a tagged antibody and the analyte to be assayed. Such antibodys are raised to specifically react with the particular analyte and may be tagged in a radioactive, fluorescent, chemluminacent, enzymatic or other manner. However, many antibodies have cross-reactions with materials other than the specific analyte to be assayed whereupon the results of simple antibody-analyte tests become unreliable. To overcome this problem, sandwich type assay techniques have been developed which utilize two antibodies to sandwich the analyte therebetween.

Conventional sandwich techniques utilize three types of assays. In the first type, the undesired cross-reaction is reduced based on the observation that the undesirable cross-reactant would not react, at the same time, with antibodies specifically raised

in two different animal species. Both of the antibodies are therefore raised against the specific analyte of interest, however they differ in that they are raised in different animal species, such as human beings and guinea pigs. Utilizing this sandwich technique, the undesirable effect of the unwanted cross-reactions of the first antibody is reduced through the utilization of the second antibody, whereby an accurate test for the analyte of interest is obtained. This type of sandwich assay technique is used in the detection of hepatitis.

The second type of sandwich assay is used when the serum contains, as metabolic by-products, fragments of the analyte to be measured. Two antibodies used for this assay are raised specifically against the analyte of interest. However, one antibody reacts specifically with one portion of the analyte while the second antibody reacts specifically with another portion of the analyte. This prior art immunometric sandwich technique thus utilizes antibodies that are specifically raised against the specific analyte to be measured. The unwanted cross-reactions of the specific antibody with a fragment of the analyte of interest have caused researchers to develop this two site immunoassay sandwich technique which minimizes the effects of the unwanted cross-reactions from a fragment of the analyte.

The third sandwich technique uses the same specific antibody twice to detect the analyte of interest. In this technique an antibody which is specific to the analyte of interest is immobilized on a solid-phase and reacted with the analyte. All other interfering substances are removed before the analyte is reacted once again

with the same antibody except that the antibody is now tagged for assay purposes.

The subject of the present invention is the use of the cross-reactive antibody sandwich technique in which enzyme immunoassay is used rather than radio-immunoassay.

The utility of cross-reactive antibodies for specifically measuring an analyte is examplified by describing a kit for measuring CK-MB isoenzyme. Several immunological and non-immunological methods for measuring CK-MB have been described, e.g., U.S. Patent Nos. 3,932,221; 4,012,285; 4,067,775 and 4,237,219. However, they do not describe a sandwich method for measuring only CK-MB.

U.S. Patent No. 4,353,982, described a triple antibody technique for measuring CK-MB by using an immunoprecipitation technique. The subject of the present invention is similar to that U.S. patent in the use of two antibodies which are not specific to CK-MB. However, the present invention discloses the use of an enzyme label in a matrix aided sandwich technique utilizing cross-reactive antibodies in which CK-MB is specifically and accurately measured at speed and freedom from interference from CK-MM, CK-BB and other isoenzymes of CK to the extent not possible by teachings of U.S. Patent No. 4,353,982. This is achieved by incorporating steps to minimize the effect of unusually large amounts of CK-MM or CK-BB and accelerating the speed at which reactions take place through the use of reaction matrix.

The prior art describes many ways of immobilizing specific antibodies on solid-phases such as those

described in U.S. Patent Nos. 3,646,346; 3,980,764; 3,980,765; 4,059,685; 4,066,512 and 4,071,409. However, immobilization of cross-reactive antibodies on the solid-phase for use in the sandwich technique has not been previously described.

The use of various enzyme immunoassay techniques involving specific binding proteins (antibodies) have been disclosed in the prior art such as described in U.S. Patent Nos. 3,654,090; 3,791,932; 3,839,153; 3,850,752 and 3,879,262. However, the use of an enzyme immunoassay technique in conjunction with cross-reactive antibodies has not been described in the prior art.

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As will be seen from the description of the preferred embodiment hereinbelow, the present invention differs from the prior art in that it specifically utilizes the previously avoided cross-reactive capabilities of two different antibodies to create a specific sandwich assay technique wherein an enzymatic label is utilized.

SUMMARY OF THE PRESENT INVENTION

It is therefore a primary objective of the present invention to provide an immunoassay technique which does not require the raising of an antibody which is specific to the analyte to be assayed.

It is another object of the present invention to provide a two site cross-reaction immunoassay sandwich technique which utilizes the cross-reactions of two different antibodies to provide a specific test for an analyte of interest utilizing an enzymatic label.

It is a further object of the present invention to provide an immunoassay technique for determining the presence of CK-MB in human serum utilizing an enzymatic label.

It is yet another object of the present invention to provide a two site cross-reaction immunoassay sandwich technique for the detection of CK-MB which utilizes the presently available antibodies for CK-MM and CK-BB and an enzymatic label.

The immunoassay technique of the present invention includes the selection of two different antibodies each of which is specific to a different analyte but each of which will cross-react with the analyte of interest. The first such antibody is affixed to a solid-phase and then reacted with the unknown sample. After reaction, the reactants are

separated and the solid-phase portion thereof is retained. The second antibody, being tagged, is then reacted with the retained solid-phase portion. After reaction, the reactants are separated and the solid-phase portion is tested for the presence of the tag. Calibration tests are conducted concurrently with known amounts of the analyte of interest. The results of the test for the unknown can be compared therewith to provide a qualitative and quantitative determination of the presence of the analyte of interest. This invention particularly utilizes an enzymatic label.

With particular regard to the detection of CK-MB the immunoassay technique of the present invention includes reacting the unknown human serum with CK-BB antibody which is affixed to a solid-phase. After the reaction is complete, the reactants are washed, centrifuged and the liquid portion thereof is aspirated. The solid-phase portion, having been retained, is then reconstituted in solution containing tagged CK-MM antibody and reacted therewith. Upon completion of the reaction, the reactants are washed and centrifuged and the liquid portion is aspirated. The retained solid-phase portion is now tested for the presence of the tag. Calibration tests are also conducted utilizing serum having known amounts of CK-MB to provide a background with which to determine the quantity of CK-MB in the unknown sample.

A primary advantage of the present invention is that an antibody that is specific to the analyte of interest need not be created in order to provide an immunological assay test for the analyte. All that is required is that there be found two different antibodies which mutually cross-react only with the analyte. The technique is very useful for those

analytes which are unstable and hence cannot be practically used to make antibodies. It is also very useful when it is extremely difficult to purify the analyte, as it is the case with cancer antigens.

It is another advantage of the present invention, as applied to the testing for CK-MB, that it utilizes the antibodies to CK-MM and CK-BB which are commercially available and inexpensive.

It is a further advantage of the present invention, as applied to the testing for CK-MB, that the test may be performed rapidly and with greater accuracy than test procedures presently available utilizing an enzymatic label.

These and other objects and advantages of the present invention will no doubt become apparent to those of ordinary skill in the art after having read the following detailed description of the preferred embodiments which are illustrated in the several figures of the drawing.

IN THE DRAWING

Fig. 1 is a flow chart depicting the two site cross-reaction immunometric sandwich assay method of the present invention;

Fig. 2 is a schematic diagram of the assay method of the present invention;

Fig. 3 is a flow chart depicting the assay method of the present invention as applied to the detection of CK-MB;

Fig. 4 is a flow chart depicting an alternative embodiment of the assay method of the present invention.

Fig. 5 is a calibration curve for an enzyme labeled test.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In standard sandwich assay techniques, two

antibodies are utilized, each of which is raised against the specific analyte of interest. The utilization of the two specific antibodies gives a very high probability of detection of the analyte and a very low probability of error due to unwanted cross-reactants. However, such sandwich techniques cannot be utilized where an antibody that is specific to the analyte of interest has not been created or isolated. The present invention has application in just such a circumstance.

Where it has proven difficult to create and/or isolate a specific antibody for the detection of an analyte of interest, it may well be the case that known antibodies exist which, while specific to other analytes, will mutually cross-react only with the analyte of interest. Where two such antibodies exist, each being specific to a different analyte but each being capable of cross-reacting with the analyte of interest and there being no other analyte to which they both will cross-react, the test procedure of the present invention will permit detection and assay of the mutually cross-reacting analyte of interest. It will be seen that just a situation exists with regard to the immuno-logical assay for creatine phospho-kinase-MB (CK-MB).

As depicted in Figs. 1 and 2, the general test procedure of the present invention is performed on a serum sample 12 containing three related analytes which are schematically depicted as a diamond shaped analyte 14, hereinafter referred to as AA, a circular shaped analyte 16, hereinafter referred to as CC, and a hybrid analyte 18 of the two other analytes, shown as having both a partial diamond shape and a partial circular shape, hereinafter referred to as AC. It

will be assumed that antibodies exist which are specific to AA, hereinafter referred to as anti-AA, and to CC, hereinafter referred to as anti-CC, but that the antibody to AC is unstable and therefore non-existant for testing purposes. However, both anti-AA and anti-CC will cross-react with AC due to the similarity of molecular structure of the A and C sites of the AC molecule to the AA and CC molecules.

In an initial procedure, which is performed prior to or concurrent with the first reaction step described hereinbelow, anti-AA 19 is affixed to a solid-phase 20 using a standard immunological procedure therefor. Also, in a separate immunological procedure performed prior to the instant test, anti-CC is tagged for later detection. Procedures for tagging antibodies are well known and radioactive, fluorescent, chemluminescent, enzymatic or other types of tags are appropriate for this test procedure.

In the first reaction step 22, the serum 12 is combined with the solid-phase anti-AA 20 and reacted 22 therewith for an appropriate time and temperature. The time and temperature will vary with the particular antibodies and analytes involved in the test procedure, as well as the concentrations thereof. In the reaction the solid phase anti-AA 20 immunochemically binds 23 with the AA 14 and removes all of the AA from the serum as it is specific to that analyte. Additionally, because the anti-AA 19 will cross-react with the AC 18, all of the AC is also removed from the serum.

In the next step of the test procedure, the reactants are separated 24, the solid-phase products 26 being retained and the liquid 28 being discarded.

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Standard separation procedures, such as centrifugation and aspiration, are utilized. It is to be noted that the discarded liquid 28 contains all of the CC analyte.

The retained solid-phase 26 is now reacted 32 with the tagged anti-CC 30. The reaction 32 is allowed to continue for an appropriate time at an appropriate temperature. Again, the time and temperature will vary with the particular antibodies and analytes involved in the test procedure, as well as the concentrations thereof.

Thereafter, a standard separation step 34, is performed wherein the solid-phase 36 is retained and the liquid 38 is discarded. As is schematically shown in Fig. 2, the tagged anti-CC 30 can only cross-react 39 with the AC which previously cross-reacted with the solid phase anti-AA 26; all of the CC having been discarded in the liquid portion 28 of the first separation step 24.

It is now seen that the remaining solid-phase 36 contains a tagged sandwich of molecules 40 in which the AC (the analyte of interest) is sandwiched between two antibodies, neither of which is specific to that analyte but each of which will cross-react with it.

The solid-phase 36 may now be tested 42 for the tag, which test will give an indication of the presence of the analyte of interest.

Calibration tests utilizing known amounts of the analyte of interest and the above-described test procedure are conducted simultaneously to create a calibration curve against which to guage the results of the test for the unknown sample. Further tests utilizing known dilutions of the unknown sample

and the above-described test procedure may also be performed to obtain test results which fall on sensitive portions of the calibration curve.

A specific application of the above-described test procedure for use in the detection of CK-MB antigen in human serum can now be described.

As is well known, human serum contains three creatine phospho-kinase isoenzymes; creatine phospho-kinase-MM (a skeletal tissue extract, hereinafter referred to as CK-MM), creatine phospho-kinase-BB (a brain tissue extract, hereinafter referred to as CK-BB) and creatine phospho-kinase-MB (a heart tissue extract, hereinafter referred to as CK-MB) which is a hybrid form of CK-MM and CK-BB. A great deal of research has been conducted to develop a test for CK-MB in that its presence is a specific indication of myocardial infarction or similar heart disturbances. However, testing for CK-MB by various methods has proved to be quite difficult as its properties are quite similar to CK-MM and CK-BB, the presence of which can significantly mask small amounts of CK-MB. With particular regard to immunometric assay techniques for the detection of CK-MB, these have been made difficult by the present inability of researchers to prepare and develop a stable antibody which is specific to the CK-MB (hereinafter referred to as anti-CK-MB), and no such anti-CK-MB is commercially available at this time. However, it is known that the antibodies to both CK-MM and CK-BB (hereinafter referred to as anti-CK-MM and anti-CK-BB respectively) will cross-react with CK-MB, in addition to their having specific reactions with CK-MM and CK-BB respectively. The test procedure described hereinabove is therefore applicable to the

detection of CK-MB in accordance with the following description.

Prior to the actual assay of serum, anti-CK-BB is affixed to a solid phase as is described hereinbelow. However, as is also described hereinbelow an alternative embodiment of the above-described test procedure may be performed in which this step is not performed. Additionally, prior to assay the anti-CK-MM must be tagged, such as is described hereinbelow by the utilization of radio-active iodine (^{125}I).

Anti-CK-BB (rabbit) for affixation to a solid-phase is commercially available from many sources. The solid-phase selected for linkage with the anti-CK-BB was Bio-Rad Immuno-Beads (catalog no. 170-5602) although other solid phases could be utilized such as latex beads or cellulose beads or a membrane strip. Bio-Rad Immuno-Beads are polyacrylamide beads having a diameter of approximately 10 μ (microns) and which are designed for use as a solid phase in immunoassay testing. Following the procedure recommended by the manufacturer, the beads were first covalently linked to goat anti-rabbit Igg. 50mg of beads were then suspended in 25 to 50ml of PBS containing 10% calf serum, although other proteins could be used to minimize non-specific binding. Thereafter, the beads were washed with PBS buffer (pH approximately 7.4, 0.02M PO_4, 0.15M NaCl). The beads were then slurried in 25 ml PBS containing 1% BSA. Approximately 100 μl of anti-CK-BB was then reacted with the beads to link the anti-CK-BB to the beads. The antibody coated beads were then washed and separated from unreacted anti-CK-BB with PBS buffer. The anti-CK-BB coated beads were then ready for utilization in the test

procedure.

Prior to testing, the anti-CK-MM is iodinated with radioactive iodine (125_I) to serve as a tag for the detection of CK-MB. Such tagging procedures are well known, see FRAKER, T. and SPECK, J., Biochemical and Bio-physical Research Commission, volume 80, no. 4, February, 1978. In the procedure, 100µg of anti-CK-MM (goat) was dissolved in 100 µl of water. Sixteen µl of this solution, to be used for tagging, was combined with 20 µl (0.25M PO_4) PBS buffer (pH, 7.5) in a reaction vial. 480 microcurie of NaI^{125} was added to this buffer. This was followed by 10µl (3.5mg/ml in 0.05 phosphate buffer) of chloramine-T solution. This was mixed for 60 seconds followed by 10µl of sodium metabisulfite solution (3.5mg/ml 0.05 phosphate buffer). The reaction mixture was fractionated over a Sephadex G-50 column (washed and treated with goat serum), fractions numbered 18-23 were collected and pooled. The fraction pool was purified over a Dowex 1x8 column and collected. This material was diluted to achieve an appropriate activity level.

Utilizing the previously prepared solid-phase anti-CK-BB and radiodinated anti-CK-MM, the test procedure for CK-MB is performed as depicted in Fig. 3. Human serum 62 containing the isoenzymes CK-MM 64, CK- MB 66 and CK-BB 68 is reacted 72 with the anti-CK-BB coated Immuno-Beads 70. The reaction time and reaction temperature being parameters which may be varied depending upon the concentration of the serum and the strength of the anti-CK-BB on the Immuno-Beads. As is demonstrated in Example II hereinbelow, these variables may be adjusted such that good test results are obtainable utilizing room

temperature and a reaction time of one hour.

Following the reaction phase 72, it is necessary to separate the liquid from the beads. To accomplish this, approximately 2ml of additional PBS buffer is added to the reactants as a wash 74. The reactants are then centrifuged 76 to achieve a button-like precipitate 78. Centrifuging at room temperature for approximately ten minutes at a speed of approximately 2000 rpm has been found to yield good results.

Having achieved a button-like precipitate 78 at the bottom of the test vial the liquid 80 therein is aspirated 82 and discarded, and the precipitate 78 is retained for the second phase of the test procedure.

It is noted that the anti-CK-BB coated beads will have reacted with, and therefore removed from solution, all of the CK-BB and CK-MB antigens. As the CK-MM antigen does not react with the anti-CK-BB, the CK-MM remains in solution and is discarded with the aspirated waste water 80.

The second reaction phase 86 of the test procedure is now initiated by the addition of the radioiodinated anti-CK-MM 88 to the precipitate. The reactants are vibrated or vortexed to reconstitute 84 the precipitate into the reaction. The reaction time and reaction temperature are parameters which may be varied in accordance with the concentrations of the reactants. As is demonstrated in Example II good test results may be obtained with the utilization of room temperature and a reaction time of approximately 1 hour.

After reaction 86, a second separation is performed in the same manner as the prior separation. That is, approximately 2ml of PBS wash buffer 90 containing 0.1% Tween 20 is added to the reactants

and the reactants are centrifuged 92 at room temperature for an approximate time of ten minutes at an approximate speed of 2000 rpm to yield a button-like precipitate 94 at the bottom of the test vial The liquid 96 in the test vial is then aspirated 98 and discarded in an appropriate manner, in that it contains excess radioactive anti-CK-MM. The button-like precipitate 94 is then tested 100 with a scintillation detector to indicate the presence of radiation as a possible indication in the test serum.

It is important to note that as all of the CK-MM was aspirated 82 and discarded in the liquid 80, there was no CK-MM present in the second reaction 86 with which the anti-CK-MM could specifically react. The only reaction possible for the anti-CK-MM was then to cross-react with the CK-MB that was bound to the beads by virtue of its cross-reaction with the anti-CK-BB.

As with all radioimmunoassay techniques, it is necessary to perform concurrent calibration tests with known amounts of CK-MB to form a calibration curve against which to compare the results of the unknown test. It is noted that there will always be some radiation from a zero level CK-MB sample due to the attachment of the radioiodinated anti-CK-MM to the walls of the test vial and remaining in any moisture within the precipitate 94 after centrifuging 92. Thus, there will be radiation from a zero level CK-MB sample and it is the increase in radiation over the zero level of radiation that provides an indication of the presence of CK-MB in the test sample.

Furthermore, it is well known in radioimmunoassay testing that the increase in radioactivity does

not vary linearly with increasing amounts of analyte in the test serum. The variation of radiation with concentration of analyte depends upon many variables, such as quantity of antibody, dilution of antibody, antibody-analyte ratio and others. The standard test procedure which is utilized to overcome this testing impediment is to perform a series of tests with varying known dilutions of the unknown test serum. A series of results are then obtained, one or more of which will lie on a sensitive portion of the calibration curve to give an accurate indication of the level of analyte in the sample. The Examples I, II and IV given hereinbelow demonstrate the utilization of this procedure.

As would be obvious to those skilled in the art, alternative solid-phase materials such as a membrane filter could be utilized instead of the Immuno-Beads. In exploring this alternative, a membrane 120 was created utilizing Whatman no. 1 filter paper which was oxidized using the periodate technique described by Ferrung, B., Maiolini, R., and Masseyeff, R., Journal of Immunological Methods, Volume 25, page 49, 1979. Strips of the membrane were soaked in goat anti-rabbit I_gG solution and utilized in much the same manner as the immuno-beads are used in the test. The use of membrane as the solid-phase eliminates the need to centrifuge, and the separation steps become the simple washing of the membrane to remove excess immunoreagents. Results using a membrane solid-phase are presented in Example VI hereinbelow.

A variation on the above described test procedure, see Fig. 4, may be conducted in the following manner. Rather than affixing the first antibody 130

to a solid-phase 132, the first antibody 130 is added 136 directly to the unknown serum 134. Thereafter, the solid-phase 132, which has not been coated with the first antibody 130, but which is prepared for coating as described hereinabove, is added 138 to the reactants. The reaction 138 is then permitted to proceed in the manner described hereinabove and the remaining steps of the test procedure are conducted as previously described following reaction 72 of Fig. 3. It is found that sufficient amounts of the solid-phase antibody-antigen complex attach to the beads 132 to give adequate test results. Examples IV and V presented hereinbelow are specific examples of the results achieved utilizing this variation of the test procedure.

The following examples demonstrate the instant testing method using several combinations of test conditions.

EXAMPLE I

This experiment was carried out with anti-CK-BB coated immuno-beads. 100μl of sample was mixed with 200μl of beads. After overnite incubation at room temperature, 0.5 ml PBS buffer with 10% calf serum was added to the test tube. The beads were separated by centrifugation. They were then reacted for 5 hours at room temperature with radioiodinated anti-CK-MM. After incubation, beads were washed with 1 ml PBS buffer with 10% calf serum. The beads were then centrifuged and counted.

Results

Normal human serum count (NHS) -- 1090 counts per min.

Calibrators (100 µl per test)	Ratio $\frac{\text{Sample Counts}}{\text{NHS COUNTS}}$
E. 1 µg/ml of CK-MB powder in serum	1.17
D. 10 µg/ml of CK-MB powder in serum	3.20
C. 50 µg/ml of CK-MB powder in serum	5.71
B. 100 µg/ml of CK-MB powder in serum	6.65

The calibrator samples were made by dissolving a powder containing 6-8% CK-MB in normal human serum at various dilutions.

Patient Samples (known to have high level of CK-MB)

High level 10 µl	3.66
25 µl	7.44
50 µl	12.1
100 µl	14.9

As is seen, a good response curve was obtained with CK-MB calibrators, and patient sample, which was identified as having high CK-MB content by other techniques, could be measured utilizing four dilutions to assure a reading level that was correlatable on the calibration curve.

EXAMPLE II

This example shows effect of reducing incubation time and varying the amount of beads to 200 µl and 500 µl. The wash buffer used was 2 ml PBS with 5% calf serum and was added before each centrifugation. The incubation time was one hour for each of the reactions at room temperature. Other test conditions were identical to those of Example I.

Results

	200 μl Beads	500 μl Beads
	Counts (cpm)	
NHS	913	1475
	Ratio $\frac{\text{Sample Counts}}{\text{NHS COUNTS}}$	
High 50 μl	2.35	2.85
100 μl	3.31	3.77
Calibrater 50 μg/ml	2.24	3.25
100 μg/ml	2.8	4.50

The ratios obtained, although significantly lower than the overnite incubation, were still quite acceptable. The effect of reduction in incubation time thus can be compensated by increasing the amount of beads.

EXAMPLE III

The method described in Example II was used with 200 μl of beads and an incubation time of 1.5 hours for each reaction. The tracer used in this experiment had higher non-specific binding. Several serial samples of a patient just admitted to a hospital for a myocardial infarct were tested.

Sample	Count
NHS	3835 counts per minute
	Ratio $\frac{\text{Sample Counts}}{\text{NHS COUNTS}}$
Calibrators 50 μg/ml	2.2
100 μg/ml	2.7

PATIENT A:	
11 A.M.	2.24
12 NOON	2.75
1 P.M.	3.00
4 P.M.	3.15
8 P.M.	3.54
NORMAL PATENTS:	
B	.94
C	1.06
D	.99

The rising levels of CK-MB shown in this Example demonstrate the well known increase in CK-MB levels in the patient following a myocardial infarct or similar incident.

EXAMPLE IV

In this example, polyacrylamide beads were used as a precipitating antibody. In the first step, diluted (1:30) anti-CK-BB was added to the sample. Bio-Rad Immuno-beads were suspended in 50 ml PBS buffer with 1% BSA, 500 µl of these beads being comparable in number of beads to 250µl of beads used in previous examples. These beads were not precoated with anti-CK-BB. After a one hour incubation period, the reactants were centrifuged and aspirated. A second incubation of one hour was carried out in the normal manner. Therafter, 2 ml of PBS buffer with 1% BSA and 1% Tween was added and reactants were centrifuged, aspirated and the solid-phase counted. The results were as follows:

Sample	Count	
NHS	2076	
	Ratio	Sample Counts / NHS COUNTS
Calibraters 50 μg/ml	3.1	
100 μg/ml	4.2	

The above sample contained roughly 5 mIU/ml and 10 mIU/ml CK-MB which is roughly the limit of normal range.

EXAMPLE V

Negative samples were run using the method discussed in Example IV.

RESULTS

	SAMPLE	COUNTS	Standard Deviation
RUN 1	NHS	2015	
	13 negative samples	2260	305
RUN 2			
	NHS, 16 times	2017	199
	12 negative samples with high total CK	2172	300
	Calibrater 50 μg/ml	4281	
	100 μg/ml	5936	

Thus negative samples, even those with high total CK levels, would all be well under the 50 μg/ml calibrator sample and are seen to be quite close to the NHS samples.

EXAMPLE VI

The use of a membrane as a solid-phase is

demonstrated by using anti-CK-BB immobilized on Whatman no. 1 filter paper as described hereinabove.

The antibody coated membrane (3/8" x 1") was placed in a mixture of sample and 2ml PBS 5% BSA buffer. After a first incubation of 1 hour, the liquid was aspirated and the membrane was thoroughly washed. Radioiodinated anti-CK-MM was added. After a second incubation of one hour the liquid was aspirated. The membrane was washed thoroughly and transferred to another tube. The results were as follows:

RESULTS

Sample		
NHS	967 counts per minute	
	Ratio	Sample Counts / NHS COUNTS
Calibrator 400 μ g/ml	3.3	
100 μ g/ml	1.9	

Thus, a membrane solid-phase is seen to produce cognizble results although they are not as pronounced as were the results utilizing the beads as the solid-phase.

It can therefore be seen that the present invention derives its uniqueness from the utilization of the cross-reactive capabilities of the antibodies that are selected for use. This is in contradistinction to standard immunological assay techniques in which the cross-reactive capabilities of the antibodies are minimized or avoided through the use of multiple antibodies which are each specific to the analyte of interest. Utilization of the instant test

technique for the detection of CK-MB in human serum provides a rapid and effective means for the detection and identification of myocardial infarcts and similar heart disturbances in patients immediately following their occurance.

The above-described methods can be conducted utilizing an enzymatic label in place of the radioactive iodine label. Such a test method has now been developed by Inventor Shah which utilizes polystyrene macrobeads, having a diameter of approximately 8 millimeters, and Horse Radish Peroxidase (HRP) as a labeling enzyme. In this enzymatic label test procedure as applied to a test for CK-MB, the CK-BB and CK-MM isoenzymes utilized to create appropriate antibodies as well as the anti-CK-BB and anti-CK-MM were produced according to methods described in the article <u>IMPROVED RADIOIMMUNOASSAY FOR CREATINE KINASE ISOENZYMES IN PLASMA,</u> authored by Ritter, Mumm and Roberts, Clinical Chemistry, Volume 27, No. 11, 1981, Page 1878, et seq..

Prior to conducting the test, the macrobeads are coated with anti-CK-BB. The procedure to accomplish the coating of the macrobeads utilizes goat anti-rabbit antibody which is diluted 1:50 in Bicarbonate-Carbonate buffer, pH 9.6. The diluted antibody was added to the macrobeads (approximately 250 ul of diluted antibody per bead) and allowed to stand at room temperature overnight. The beads were than removed from the antibody solution. Rabbit-anti-CK-BB was prepared by adding the antibody to Tris-Acetic acid buffer containing 0.1% BSA plus 3% PEG; the dilution of the antibody depends upon the titer of

the antibody and such dilution is performed so that approximately 250 ul of the antibody solution provides more than enough rabbit antibody to fully bind all of the goat anti-rabbit antibody previously bound to the macrobeads. The macrobead solution was then incubated for 24 hours at 2-6 degrees Centrigrade. Thereafter, the excess rabbit-anti-CK-BB solution was removed and the beads were washed a first time with Tris-Acetic acid buffer container 1% BSA and then washed a second time with Tris-Acetic acid buffer containing 0.0025% Gluteraldehyde.

Prior to conducting the test procedure, goat anti-CK-MM was enzyme labeled utilizing Horse Radish Peroxidase (HRP). In conjugating HRP to anti-CK-MM, 5mg of HRP was dissolved in 1.0 ml of freshly prepared 0.3 M Sodium Bicarbonate, pH 8.1 and thoroughly mixed. Then 100 ul of 1% Fluocodinitco Benzene (FDNB) diluted in absolute Ethanol was added and mixed gently for one hour at room temperature. Thereafter, 1 ml of 0.05 M Sodium Periodate in distilled water was added and mixed gently for 30 minutes at room temperature. Thereafter, 1 ml of 0.16 M Ethylene Glycol in distilled water was added and mixed gently for 1 hour at room temperature. The above solution was then dialyzed against three one-liter changes of 0.01 M Sodium Carbonate buffer. Thereafter, anti-CK-MM antibody is dissolved in 300 ul of Carbonate buffer. The anti-CK-MM solution is then mixed with 1.3 mg of the dialyzed HRP solution for 3 hours at room temperature. Thereafter, 4 mg of Sodium Borohydrate ($NaBH_4$) is added and mixed thoroughly and left at 2-6 degrees Centigrade overnight. The mixture is then dialyzed at 4 degrees Centigrade against PBS. Thereafter, the dialyzed sample is applied to a Sephadex G-100 column

equilibrated in PBS. Approximately 1 ml fractions are collected and read at 280 and 403 NM. The fractions containing the conjugated antibody are then pooled and aliquots of the conjugate are stored in the presence of 10 mg BSA per ml of the conjugate at -20 degrees Centigrade.

The enzyme labeled test procedure is conducted utilizing the coated macrobeads and the conjugate. First, 200 ul of sample to be tested is added to a test tube with a coated macrobead and incubated for 30 minutes. Thereafter, 2 ml of wash buffer is added and then aspirated. Thereafter, 200 ul of conjugate is added to the bead and incubated for 30 minutes. Three wash steps are then conducted wherein 2 ml of wash buffer are added and then aspirated. This washing is important in obtaining consistant and accurate results. Following the third aspiration, 200 ul of OPD Buffer are added and incubated for 30 minutes. The incubation is then haulted by adding 1 ml 1N H_2SO_4. The enyzme reading is then taken at 490 NM.

As is described hereinabove, several calibrators with known differing concentrations of CK-MB are simultaneously tested such that a calibration curve against which the unknown sample test results can be compared is created.

EXAMPLE VII.

Table I presents results utilizing the above-described enzyme labeled test procedure for "Sample A" and 5 calibrators (A, B, C, D, E) and a control. The test was run twice and an average reading calculated. Figure 5 presents a calibration curve of the results.

EXAMPLE VIII.

The accuracy of this enzyme labeled test

procedure and the rate of reaction can be increased by diluting the enzyme tagged conjugate in a buffer containing 2-10% PEG and 0.1% to 10% serum (bovine serum was used). The use of this dilutant minimizes the non-specific binding of the conjugate whereby accuracy is increased and the rate of reaction is accelerated. Table II provides a comparison of test results for the calibrators utilizing an enzyme conjugate formed utilizing the dilutant and not utilizing the dilutant. It can be seen that a calibration curve utilizing the dilutant data would be steeper, such that a more accurate test procedure is created by utilizing the dilutant in formulating the enzyme conjugate.

EXAMPLE IX.

In further demonstrating the accuracy of the above-described enzyme labeled test procedure, 117 patient samples were comparatively assayed utilizing a radioisotope 125I tag and the HRP enzyme tag. A close correlation of results was obtained, which could be expressed by the equation EIA = 1.08 (RIA) - 0.19. The RIA test procedure has been commercially available for approximately 3 years and has obtained acceptance in the industry. The efficacy of the EIA test is thus demonstrated.

Whereas the preferred embodiment of the present invention has been described above, it is contemplated that other alterations and modifications may become apparent to those skilled in the art after having read the above disclosure. It is therefore intended that the appended claims be interpreted as covering all such alterations and modifications as fall within the true spirit and scope of the invention.

What is claimed is:

TYPICAL DATA AND CALIBRATION CURVE FOR THE ASSAY

Table 1.

Calibrators	Concentration EU/L*	A 490nm	Average A 490nm
A	2	0.196 0.189	0.192
B	4	0.399 0.425	0.412
C	10	0.615 0.618	0.616
D	20	0.876 0.845	0.860
E	40	1.237 1.199	1.218
Control	3.3	0.359 0.341	0.350
Sample A	12.2	0.681 0.678	0.679

*One EU is that mass of enzyme which gives 1 Unit of activity as measured by Calbiochem Stat-Pak at 30°C.

TABLE II

O.D. 490 nm

CALIBRATOR	PEG AND CALIF. SERUM	WITHOUT PEG AND CALIF. SERUM
40EU/L	0.95	0.44
20EU/L	0.70	0.31
10EU/L	0.50	0.24
4EU/L	0.36	0.19
2EU/L	0.20	0.12

EFFECT OF PEG AND CALIF. SERUM ON CALIBRATION CURVE RESULTS

CLAIMS

1. An immunoassay method for the detection and measurement of a specific group of analytes of interest in a liquid sample, utilizing only non-specific and cross-reactive antibodies which comprises an assay method wherein the analyte of interest and cross-reactive antibodies have the following characteristics:

1. The analyte of interest is not specifically measureable using specific monoclonal or polyclonal antibodies, due to other molecules within said liquid sample which cross-react with specific antibodies to said analyte of interest;

2. The analyte of interest cannot be fragmented into two or more pure and stable fragments which are capable of forming specific antibodies only to said fragments;

3. The analyte of interest is a hapten or antigen or a conjugate of a hapten or antigen;

4. The analyte of interest has at least two immunochemically differing binding sites:

and wherein the analyte of interest is immunochemically separable from said liquid by reacting it with non-specific cross-reacting antibodies, neither of which antibodies is specifically produced against said analyte of interest;

and wherein each of said antibodies will cross-react with a differeing one of the differing binding sites of the analyte of interest; each of said cross-reacting antibodies being raised against an immunochemically different molecule, where each of said molecules has at least one immunochemically similar reactive site to said analyte of interest and one or

more immunochemically differing reactive sites from said analyte of interest, and wherein said molecules are not fragments derived from said analyte of interest, which includes the following steps:

(a) selecting a first antibody which will cross-react with a binding site of said analyte of interest and affixing said first antibody to a solid-phase to form a bound antibody;

(b) selecting a second antibody which differs from said first antibody and which will cross-react with a second binding site of said analyte of interest and tagging said second antibody to form a tagged antibody, said first and second antibodies being further selected such that within the test liquid only the analyte of interest has binding sites with which both of them will cross-react;

(c) reacting said bound antibody with a liquid suspected of containing said analyte of interest to form a first reaction solution;

(d) separating the solid-phase portion of said first reaction solution from the liquid portion thereof;

(e) reacting said solid-phase portion of said first reaction solution with said tagged second antibody to form a second reaction solution;

(f) separating the solid-phase portion of said second reaction solution from the liquid portion thereof; and

(g) testing said solid-phase portion of said second reaction solution to detect the tag of said second antibody as an indication of the presence of said analyte of interest;

and wherein said tag is an enzyme tag.

2. A two site cross-reaction immunometric sandwich assay method as recited in claim 1 wherein said solid-phase is a material selected from the group consisting of polyacrylamide beads, latex beads, cellulose beads, polystyrene beads and a membrane strip.

3. A two site cross-reaction immunometric sandwich assay method as recited in claim 1 and further comprising repeating steps (a) - (g) several times substituting for said serum in each repetition of steps (a) - (g) a sample containing a different known amount of said analyte whereby a calibration curve is created; and comparing the test results for said serum to said calibration curve.

4. A two site cross-reaction immunometric sandwich assay method for the assay of creatine phospho kinase-MB (CK-MB) in human serum which also contains creatine phospho kinase-MM (CK-MM) and creatine phospho kinase-BB (CK-BB), comprising:

(a) affixing an antibody to CK-BB to a solid-phase to form a bound antibody;

(b) tagging an antibody to CK-MM;

(c) reacting said bound antibody with a serum suspected of containing CK-MB to form a first reaction solution;

(d) separating the solid-phase portion of said first reaction solution from the liquid portion thereof;

(e) reacting said solid-phase portion of said first reaction solution with said tagged antibody to form a second reaction solution;

(f) separating the solid-phase portion of the

second reaction solution from the liquid portion thereof;

(g) testing said solid-phase portion of said second reaction solution to detect the tag of said antibody to CK-MM as an indication of the presence of CK-MB; and

wherein said tag is an enzyme label.

5. A two site cross-reaction immunometric sandwich assay method as recited in claim 4 wherein said solid-phase is a material selected from the group consisting of polyacrylamide beads, latex beads, cellulose beads, polystyrene beads and a membrane strip.

6. A two site cross-reaction immunometric sandwich assay method as recited in claim 4 and further comprising repeating steps (a) - (g) several times substituting for said serum in each repetition of steps (a) - (g) a sample containing a different known amount of said analyte whereby a calibration curve is created; and comparing the test resutls for said serum to said calibration curve.

7. A two site cross-reaction immunometric sandwich assay method as recited in claim 4 wherein said step (f) includes washing said solid-phase portion to remove said liquid portion therefrom.

8. A two site cross-reaction immunometric sandwich assay method as recited in claim 4 wherein said enzyme tag is Horse Radish Peroxidase.

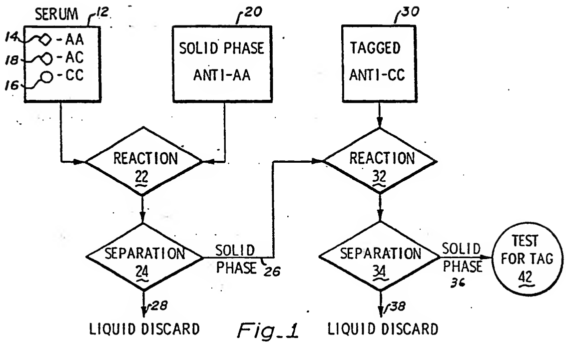

Fig-1

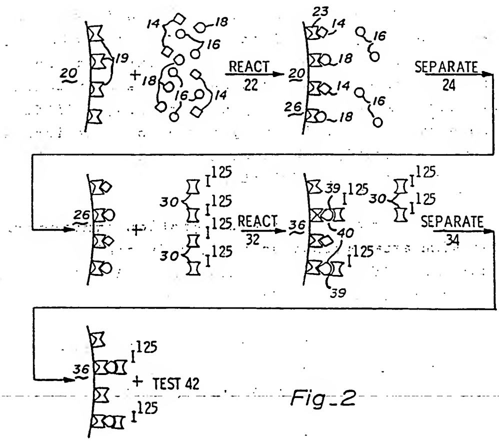

Fig-2

SUBSTITUTE SHEET

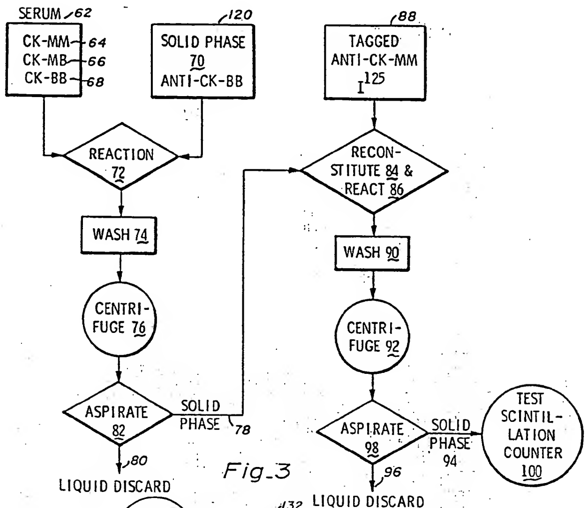

Fig_3

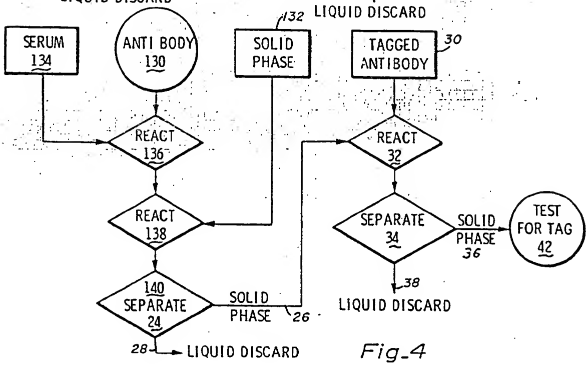

Fig_4

SUBSTITUTE SHEET

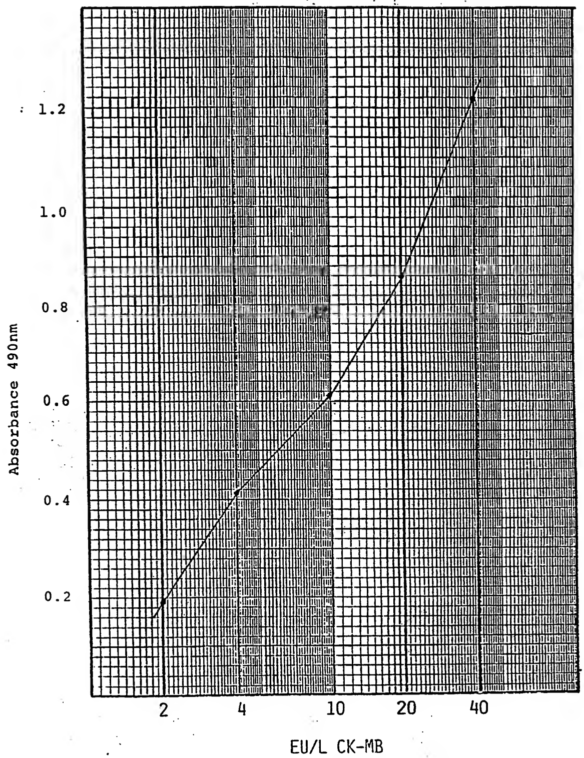

FIG. 5

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01441

I. CLASSIFICATION F SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.- 435/7
IPC.4 G01N 33/543, G01N 33/573

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	435/4,7,17,805,810 436/518,538,539,540,541,542,530,533,547

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US,A, 4,332,783 Published 01 June 1982, Pernice	1-8
Y	US,A, 4,353,982 Published 12 October 1982, Gomez	1-8
Y	US,A, 4,244,940 Published 13 January 1981, Jeong	4-8
Y	US,A, 4,237,219 Published 02 December 1980, Roberts	4-8
A	N, Clin.Chim.Acta, 83, issued 1978 Roberts et al, 141-149.	1-8
A	N, Clinical Chemistry, 27(11), issued 1981, Ritter et al, 1878-1887.	1-8

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
09 October 1985	15 OCT 1985
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Esther M. Kepplinger Esther M. Kepplinger

Form PCT/ISA/210 (second sheet) (October 1981)